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Voltage Clamp Fluorometry of P-Type ATPases

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Abstract

Voltage clamp fluorometry has become a powerful tool to compare partial reactions of P-type ATPases such as the Na^+, K^+ -ATPase and H^+, K^+ -ATPase with conformational dynamics of these ion pumps. Here, we describe the methodology to heterologously express membrane proteins in *X. laevis* oocytes and site-specifically label these proteins with one or more fluorophores. Fluorescence changes are measured simultaneously with current measurements under two-electrode voltage clamp conditions.

Keywords

Fluorescence; Distance constraints; Donor photobleaching

1 Introduction

Voltage clamp fluorometry has become a facile method to detect site-specific conformational changes of membrane proteins in real time under physiological conditions. This is achieved by combining fluorescence and two electrode voltage clamp (voltage clamp fluorometry). In contrast to other approaches, voltage clamp fluorometry can be used to obtain time-resolved and spatially well-defined information on conformational rearrangements of membrane proteins. Voltage clamp fluorometry has been used to investigate the conformational dynamics of K^+ channels, Na^+ channels, and glutamate transporters [1–4]. Equally, this approach has also been used to elucidate distance constraints for multiple membrane proteins [5, 6]. More relevant to this book, voltage clamp fluorometry was initially used in the P-type ATPase field to study the conformational dynamics of the extracellular loop between transmembrane five and six of the alpha subunit of the Na^+, K^+ -ATPase [7]. Analysis of these experiments eloquently demonstrated that fluorescence intensity changes measured after site-specific fluorescence labeling within this loop could be used to investigate the conformational state and kinetics of the Na^+, K^+ -ATPase. Analysis of subsequent experiments examined the conformational dynamics of the alpha, beta and gamma subunits as a function of ion transport as well as elucidated distance constraints between the three subunits of this ion pump [8–11]. More recently, voltage clamp fluorometry was used to examine the electroneutral H^+, K^+ -ATPase [12, 13]. Combined, analyses of these experiments have provided new information about site-specific molecular movements of P-type ATPases during ion transport.

The methods underpinning this approach are multifaceted. First, the gene of interest is inserted into a plasmid which can be translated by *X. laevis* oocytes. All free extracellular cysteine residues are removed from the target protein and a single cysteine residue is inserted at the interface of the transmembrane-extracellular domains as predicted by hydropathy analysis or through inspection of crystal structures. mRNA is synthesized *in vitro* from this plasmid and injected into oocytes isolated from *X. laevis*. Oocytes are incubated for 2–7 days at 18 °C to enable protein synthesis and surface expression. Surface expressed protein is then site-specifically labeled with one or more fluorophores. Surface expression of membrane proteins can be validated with biotinylation of the expressed proteins with subsequent Western blotting with an antibody specific for the protein of interest [14]. Functional expression of the target protein is validated using a two electrode voltage clamp set-up. Changes in fluorescence intensity are measured concurrently with ion transport using a fluorescence microscope with a tungsten lamp, filters, and photomultiplier tube upon activation of the protein of interest. Finally, while not utilized for the investigation of P-type ATPases, it is important to note that parallel approaches to study the conformational dynamics of membrane proteins on the surface of cells have more recently included patch clamp fluorometry and the utilization of lanthanides to obtain distance constraints [15, 16]. Equally, it is possible to obtain more detailed information in regard to changes in fluorescence intensity through the use of charge-coupled device cameras [2].

Combined, voltage clamp fluorometry enables examination of changes in the conformational dynamics of P-type ATPases during ion transport. Equally, it has enabled investigation of the electroneutral ion pump H^+, K^+ -ATPase.

2 Materials

All solutions should be prepared using ultrapure water. All solutions required for DNA and/or mRNA synthesis should be autoclaved with diethylpyrocarbonate (DEPC). All reagents are analytical grade.

2.1 mRNA Synthesis

1. DNA can be dissolved in DEPC-treated H_2O . The pTLN vector has been successfully used to synthesize mRNA for functional expression in *X. laevis* oocytes [17]. With this vector, the restriction enzyme *MluI* can be used to linearize the plasmid DNA prior to mRNA synthesis as long as the restriction site is not present in the gene of interest. Following linearization, High-Pure PCR Product Purification Kit (Roche Applied Science: 11732 676 001) can be used to purify the linearized DNA.
2. mMessage mMachine kit (Life Technologies) can be used to synthesize mRNA according to the manufacturer's instructions.
3. TAE buffer: 40 mM Tris-HCl (pH 8.0–8.5), 20 mM acetic acid, and 1 mM EDTA.
4. Agarose.
5. Cell-Porator (Life Technologies).

2.2 *X. laevis* Oocytes and mRNA Components

1. Mature female *X. laevis* frogs (Nasco, Cat. No. LM00535MX) can be used with good results. Maintenance of these frogs should be in accordance with institutional requirements. Alternatively, oocytes can be purchased (Ecocyte Bioscience).
2. Anesthetic solution: 0.5–3.0 g MS-222 in water, pH 7.0–8.0 w/sodium bicarbonate.
3. Diaper pads.
4. Microdissection scissors, forceps, and hooks (World Precision Instruments).
5. 3-0 to 4-0 Ethicon Vicryl sutures (Johnson and Johnson).
6. Collagenase Type II (Worthington Biochemical). Sample batches of different collagenase type II lots can be used to probe which lot works best for oocyte digestion protocol.
7. Ringer's solution (–) Ca^{2+} : 90 mM NaCl, 2 mM KCl, 2 mM MgCl_2 , 5 mM MOPS pH 7.4.
8. Ringer's solution (+) Ca^{2+} : 90 mM NaCl, 2 mM KCl, 2 mM CaCl_2 , 5 mM MOPS pH 7.4.

2.3 Voltage Clamp Fluorometry

1. Filter sets (Semrock, Inc. or Chroma Technology Corporation).
2. Loading buffer: 110 mM NaCl, 2.5 mM sodium citrate, 10 mM MOPS/Tris, pH 7.4.
3. Post-loading buffer: 100 mM NaCl, 1 mM CaCl_2 , 5 mM BaCl_2 , 5 mM NiCl_2 , 10 mM MOPS/Tris, pH 7.4.
4. Tetramethyl-6-maleimide or fluorescein maleimide (Life Technologies).
5. RC-10 chamber (Warner Instruments).
6. Polarized filters (Linos Photonics).
7. Axio Examiner Fluorescence Microscope (Zeiss).
8. Photomultiplier tube detection system for fluorescence measurements (Cairn Research).
9. Turbo-Tec-05X amplifier (NPI Instruments).
10. Axioscope 1440 Digitizer (Axon Instruments).
11. pCLAMP Software (Axon Instruments).

3 Methods

3.1 mRNA Synthesis

1. Digest 3 μg of plasmid DNA in 50 μL DEPC-treated H_2O with an appropriate restriction enzyme (1 h, 10 U of enzyme) and ensure RNase-free conditions (wear gloves, change them frequently).

2. To purify the linearized template, use the High-Pure PCR Product Purification Kit. Here, add 350 μ L binding buffer to the digest vial and vortex.
3. Place spin columns in collection tubes, load the columns with sample and centrifuge for 20 s at full-speed (eppendorf-centrifuge, room temperature). Discard the flow through.
4. Add 500 μ L of wash buffer to the column, centrifuge for 20 s at full speed, and discard the flow through.
5. Add 200 μ L of wash buffer to the column, centrifuge for 30 s at full speed (the column should be dry) and discard the flow through.
6. Place the spin column into a new autoclaved eppendorf tube, add 50 μ L of DEPC-treated water on the column material and wait 1 min. Elute by 30 s full-speed centrifugation.
7. To concentrate the DNA solution, place the sample in a SpeedVac until the concentration of the DNA is approximately 166 ng/ μ L.
8. To synthesize mRNA *in vitro* use the mMessage mMachine Kit. Here, choose the appropriate kit according to the promoter available on the plasmid DNA (ex. SP6 or T7). Normally, a 10 μ L reaction is sufficient. Combine the following reagents: 5 μ L NTP-cap mix (usually pre-aliquoted), 3 μ L linearized template DNA solution (from **step 7**), 1 μ L 10 $\times$ transcription buffer and 1 μ L (appropriate) RNA polymerase. The sample should be incubated for 1–5–2 h at 37 $^{\circ}$ C. Do not extend the reaction beyond 2 h as the yield does not increase. Furthermore, the integrity of the mRNA might be impaired.
9. To purify the mRNA, combine 12.5 μ L LiCl solution from the kit (stored at -20° C) and 15 μ L DEPC treated water (also from the kit). Mix briefly, do not vortex, centrifuge briefly, and place in -20° C 30 min or overnight for precipitation. Precool a centrifuge to $+4^{\circ}$ C. Centrifuge for 15 min at full speed (4° C). Here, a white pellet should be visible at bottom of tube. Cautiously remove the supernatant and add 150 μ L 70 % RNA grade ethanol (stored at -20° C). Centrifuge for 5 min at full speed at 4° C. Remove the supernatant completely and dry the pellet in SpeedVac briefly until all the ethanol has evaporated (2–7 min at RT). Redissolve the pellet in 12 μ L DEPC treated water.
10. To quantify the yield, load 0.3 μ L RNA solution on a 1 % agarose gel (freshly cast, fresh TAE buffer in electrophoresis chamber). mRNA should show a clear band structure (several bands are possible due to secondary structure). Degradation will show up as a broad smear. Use a NanoDrop to determine the mRNA concentration.

3.2 Isolation of *X. laevis* oocytes and mRNA Injection

1. Prior to surgery, the frog should be fasted for 12 h to prevent vomiting during the operation. To begin surgery, the frog is immersed in anesthetic solution (0.5–3.0 g MS-222 in water, pH to 7.0–8.0 with sodium bicarbonate) until the frog becomes unresponsive to a toe pinch (*see* Note 1).

2. The frog is removed from the anesthetic solution, and placed dorsal side down on the absorbent side of a diaper pad. A wet paper towel is placed on the frog to keep it moist.
3. A small coelomic incision on one side of the frog's midline is made. The ovary is identified and removed. The incision is sutured using an interrupted suture pattern. The frog is returned to its housing to recover from anesthesia.
4. The isolated ovarian lobe is cut with scissors into smaller parts in a petri dish containing Ringer's solution – Ca^{2+} . The oocytes are transferred into Ringer's Solution – Ca^{2+} with ~3 mg/mL collagenase. The sample is gently shaken in solution for 2 h at 18 °C. When most of the oocytes are separated, the oocytes are washed with Ringer's Solution – Ca^{2+} . The oocytes are washed exhaustively in Ringer's + Ca^{2+} solution and transferred to Ringer's Solution + Ca^{2+} supplemented with 1 mg/mL gentamycin.
5. The oocytes are injected with a total volume of 50 nL. For the Na^+, K^+ -ATPase, best results have been observed with 25 ng of the sheep α_1 subunit and 1 ng of the β_1 subunit. When required, 1 ng of the sheep γ subunit is injected along with the α_1 and β_1 subunits (*see* Note 2).
6. The oocytes are incubated in Ringer's Solution + (Ca^{2+}) and antibiotic (ex. 1 mg/mL gentamycin) at 18 °C in the dark for 3–7 days. This allows the protein of interest to be expressed within the oocyte plasma membrane.

3.3 Voltage Clamp Fluorometry

1. On the day of the experiment, the oocytes are removed from the incubator and placed in loading buffer for 45 min and then in post-loading buffer for 45 min to increase the intracellular Na^+ concentration to facilitate measuring the Na^+, K^+ -ATPase [11].
2. The oocytes are incubated in post-loading buffer that contains 5 μM of the desired fluorophore, such as tetramethylrhodamine-6-maleimide (TMRM) or fluorescein-5-maleimide (FM), for at least 5–10 min in the dark at room temperature. After fluorophore labeling, the oocytes are washed exhaustively with dye-free post-loading buffer and are kept in the dark until the experiment.
3. To prepare for the two-electrode voltage clamp measurements, microelectrodes are filled with 3 M KCl where the resistance of each micro-electrode is between 0.5 and 1.5 M Ω . Our laboratory uses a Zeiss Axio Examiner Fluorescence Microscope with a Cairn photomultiplier tube detection system for fluorescence measurements (*see* Note 3). In addition, the fluorescence microscope contains a 535DF50 excitation filter, a 565 EFLP emission filter and a 570DRLP dichroic mirror for TMRM.

¹This protocol has been approved by WPI's Institutional Animal Care and Use Committee.

²Our laboratory has successfully injected oocytes manually using a Nanoject Injector (Drummond Scientific) or through an automated system Roboinject (MultiChannel Systems).

³We have found the most success using an upright fluorescence microscope with water immersion lens. However, inverted fluorescence microscopes have also been effectively used [19].

4. An oocyte is placed in a RC-10 chamber on the fluorescence microscope stage. The oocyte is then impaled by the microelectrodes. Using the amplifier to hold the membrane potential at a constant value, ion flux across the membrane is measured following solution exchange or by changing the membrane potential. For concurrent fluorescence measurements, a 100 W tungsten light source equipped with an emission filter excites the fluorophore and a photomultiplier tube detects changes in fluorescence intensity (*see* Fig. 1). Measurements under Na^+/Na^+ -exchange conditions have been performed in Na^+ test solution (100 mM NaCl, 5 mM BaCl_2 , 5 mM NiCl₂, 10 μM ouabain, 5 mM Mops, Tris, pH 7.4) (*see* Note 4). For Na^+/K^+ titration experiments and stationary pump current recordings, 0.1–10 mM K^+ have been replaced for equimolar amounts of Na^+ . For K^+/N -methyl-d-glucamine (NMDG) titration experiments, 0.1–1 mM K^+ have been combined with NMDG for a final ionic concentration of 100 mM. Heterologously expressed Na^+,K^+ -ATPase was largely inhibited by 10 mM ouabain due to two mutations (Q111R and N122D) on the α subunit, which confers a lower binding affinity for the heterologously expressed Na^+,K^+ -ATPase when compared to the endogenous *X. laevis* oocyte Na^+,K^+ -ATPase [18].
5. To measure distance constraints, two accessible extracellular cysteine residues are labeled. Here, the oocytes are labeled in post loading buffer containing 1 μM FM (the donor fluorophore) and 4 μM TMRM (the acceptor fluorophore) for 30 min on ice in the dark, or only 1 μM FM. This allows for measurements to be made of holoenzymes labeled with and without an acceptor fluorophore. For these experiments, the following filter set is used: 525AF45 excitation filter, 565ALP emission filter, and 560DRLP dichroic mirror. At these concentrations and with the assumption that both fluorophores react with the free cysteine residues at the same rate, it is predicted that cysteine residues will be labeled in the following ratios: 4 % FM–FM, 32 % with TMRM–FM, and 64 % with TMRM–TMRM (*see* Note 5). Maintaining a continuous solution flow, the time dependence of donor bleaching is measured in the presence and absence of the acceptor fluorophore (*see* Note 6 and Fig. 2). These results can be used to calculate the distance between two residues on the Na^+,K^+ -ATPase utilizing Förster's equation (*see* Note 7).

⁴*X. laevis* oocytes have an endogenous Na^+,K^+ -ATPase which can be inhibited upon addition of 10 μM ouabain. The sheep Na^+,K^+ -ATPase used in our studies contains two mutations within the α_1 subunit (Q111R and N122D) which confers a reduced ouabain sensitivity (mM range) [18]. Therefore, the endogenous ion pump can be selectively inhibited with 10 μM ouabain and the heterologously expressed ion pump can be inhibited upon addition of 10 mM ouabain.

⁵Under these conditions, for the donor photobleaching experiments, the major fluorophore pair will be FM–TMRM. The TMRM–TMRM pair is not measured due to the selection of excitation/emission filters. There will be a small population of FM:FM pairs. This will result in a faster observed decay rate and this could result in a smaller distance calculated than is actually present.

⁶A continuous solution flow is required as the light source generates heat which may result in heat-induced fluorescence changes. The constant solution flow maintains constant temperature during the experiment. Alternatively, one can use a chamber with temperature control to maintain constant temperature during the donor photobleaching experiments.

⁷To determine the distance constraints between two residues, the following protocol was used. First, the efficiency of energy transfer (E) was calculated using the following equation: $E = 1 - \tau_{\text{DA}}/\tau_{\text{D}}$ (where τ_{DA} and τ_{D} are the time constants of donor photobleaching in the presence and the absence of acceptor, respectively) [20]. Fluorescence resonance energy transfer (FRET) efficiency was converted to distance using the Förster equation: $E = 1/(1 + R^6/R_0^6)$, where R is the distance between the donor and acceptor and R_0 is the distance corresponding to 50 % efficiency for a specific donor–acceptor pair (for the fluorescein–tetramethylrhodamine pair: 55 Å) [20]. R_0 is defined by the equation: $R_0 = (9.7 \times 10^3 J \Phi_{\text{D}} n^{-4} \kappa^2)^{1/6}$ (in Å), where J is the normalized spectral overlap of the donor emission and acceptor absorption, Φ_{D} is the donor emission quantum yield in absence of the acceptor, n is the index of refraction, and κ^2 is the orientation factor for a dipole-dipole interaction [21].

6. Anisotropy is calculated alongside distance measurements to determine the range of κ^2 values (*see* Note 8). Anisotropy measures the relative mobility of the fluorophore due to rotation. This can be measured using polarized filters during fluorophore excitation and emission.
7. To measure the relative movement of protein subunits, double cysteine constructs are employed that are labeled with acceptor and donor fluorophores. For these experiments, the microscope is equipped with the following filter set: 475AF40 excitation filter, 595AF60 emission filter, and 505DRLP dichroic mirror.
8. Here, the donor is excited with a 100 W tungsten light source and the fluorescence intensity of the acceptor fluorophore is measured. Using an appropriate method such as voltage or solution exchange, changes in the distance between the two fluorophores (donor and acceptor) can be correlated to movement of the labeled residues towards or away from each other (*see* Fig. 2).

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⁸The anisotropy (r) of the donor and acceptor fluorophores can be measured on oocytes which represent a quasiplanar system and evaluated through the use of the following equation: $r = (I_{\parallel} - I_{\perp}) / (I_{\parallel} + 2I_{\perp})$, where $I_{\parallel}$ is the parallel and $I_{\perp}$ is the perpendicular-emitted light with respect to the polarized excitation light at each amino acid [22, 23]. These components can be measured sequentially with polarized filters placed adjacent to the emission and excitation filters. To calculate the error in the distance measurements due to anisotropy, the following equations can be used: $\kappa^2_{\max} = 2/3 (1 + F_{rd} + F_{ra} + 3F_{rd} \times F_{ra})$ and $\kappa^2_{\min} = 2/3(1 - (F_{rd} + F_{ra})/2)$ where $F_{rd} = (r_d/r_o)^{0.5}$ and $F_{ra} = (r_a/r_o)^{0.5}$; r_d is the anisotropy of FM, r_a is the anisotropy of TMRM, and r_o is the fundamental anisotropy of each fluorophore (FM: 0.4 and TMRM: 0.38) [24, 25].

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Fig. 1.

Stationary current and fluorescence measurements of Na⁺,K⁺-ATPase α/β complexes containing different β subunit constructs upon expression in *Xenopus laevis* oocytes. **(a)** Stationary pump currents of the Na⁺,K⁺-ATPase expressed with wild-type and single cysteine mutants of the β subunit at 0 mV holding potential in response to 10 mM K⁺. Data originated from 5 to 11 oocytes; values are means ± SEM. **(b)** Parallel recording of pump current (*top*) and fluorescence change (*bottom*) from an oocyte co-injected with the sheep Na⁺,K⁺-ATPase α₁ subunit (where all extracellular cysteine residues have been replaced with conservative mutations which do not affect the function of the ion pump) and the sheep β₁ subunit with F64C mutation in response to 10 mM K⁺ and 10 mM ouabain at 0 mV holding potential. Reprinted with permission from ref. [10]. ©2005 Dempski et al. Journal of General Physiology. 125:505–520. doi: 10.1085/jgp.200409186

Fig. 2.

Distance measurements and relative movement of the α , β , and γ subunits of the Na^+ , K^+ -ATPase. (**a–c**) Normalized F_{donor} photobleaching for the Na^+ , K^+ -ATPase $\alpha\text{L306C}/\beta/\gamma\text{R19C}$, $\alpha\text{I788C}/\beta/\gamma\text{R19C}$, and $\alpha/\beta\text{P66C}/\gamma\text{R19C}$ constructs, respectively, labeled with donor fluorophore alone (black line) or donor plus acceptor fluorophore (grey line) in a 1:4 ratio. The results are the means of 4–7 oocytes $\pm$ standard error. The photodestruction rates for $\alpha\text{L306C}/\beta/\gamma\text{R19C}$ are $99.7 \pm 0.4 \text{ s}^{-1}$ for the donor only and $144.3 \pm 0.2 \text{ s}^{-1}$ for the donor in the presence of acceptor. The photodestruction rates for $\alpha\text{I788C}/\beta/\gamma\text{R19C}$ are $156.3 \pm 0.9 \text{ s}^{-1}$ for the donor only and $434 \pm 2 \text{ s}^{-1}$ for the donor in the presence of acceptor. The photodestruction rates for $\alpha/\beta\text{P66C}/\gamma\text{R19C}$ are $161.1 \pm 0.6 \text{ s}^{-1}$ for the donor only and $291.0 \pm 0.6 \text{ s}^{-1}$ for the donor in the presence of acceptor. (**d–f**) Relative movement of the αL306C residue (M3–M4 loop) of the α subunit, the αI788C residue (M5–M6 loop) of the α subunit, and the βP66C residue (extracellular–transmembrane interface of the β subunit) in relation to the γR19C residue (extracellular–transmembrane interface of the γ subunit), respectively. Parallel recording of the pump current (lower, black) and fluorescence resonance energy transfer change (upper, grey) at -40 mV under Na^+/Na^+ exchange conditions (black bars) in response to 10 mM K^+ (dark grey bars) and 10 mM ouabain (light grey bars). Results are from a single oocyte, but the experiments were repeated, with essentially identical results, on at least four occasions. The inset of each figure (in black) shows the fluorescence response to a change in membrane potential from 0 to -200 mV (arrows indicate the onset and offset of the membrane potential pulse) where the scale bars on the x and y axes are $0.01 \% F/F$ and 200 ms , respectively. Reprinted with permission from ref. [8]. Copyright 2008 American Chemical Society